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BAG5 Inhibits Parkin and Enhances Dopaminergic Neuron Degeneration

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Summary

Loss-of-function mutations in the *parkin* gene, which encodes an E3 ubiquitin ligase, are the major cause of early-onset Parkinson's disease (PD). Decreases in parkin activity may also contribute to neurodegeneration in sporadic forms of PD. Here, we show that *bcl-2-associated athanogene 5* (BAG5), a BAG family member, directly interacts with parkin and the chaperone Hsp70. Within this complex, BAG5 inhibits both parkin E3 ubiquitin ligase activity and Hsp70-mediated refolding of misfolded proteins. BAG5 enhances parkin sequestration within protein aggregates and mitigates parkin-dependent preservation of proteasome function. Finally, BAG5 enhances dopamine neuron death in an in vivo model of PD, whereas a mutant that inhibits BAG5 activity attenuates dopaminergic neurodegeneration. This contrasts with the antideath functions ascribed to BAG family members and suggests a potential role for BAG5 in promoting neurodegeneration in sporadic PD through its functional interactions with parkin and Hsp70.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disease that is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Lang and Lozano, 1998). Protein aggregates known as Lewy bodies (LBs) are pathological hallmarks of PD, and protein aggregation is a common feature among many neurodegenerative diseases (Lang and Lozano, 1998; Sherman and Goldberg, 2001). Recently, genes linked to hereditary PD have been identified, including α-*synuclein*, *DJ-1*, *PINK1*, and two components of the ubiquitin-proteasome system (UPS): *ubiquitin carboxy-terminal hydrolase L1* (*UCH-L1*) and *parkin* (*PARK2*) (Vila and Przedborski, 2004). Current evidence implicates the impaired regulation of protein aggregation and dysfunction of the UPS as a common pathway in the progression of both genetic and sporadic forms of PD (Giasson and Lee, 2003; Sherman and Goldberg, 2001).

The UPS mediates the ubiquitinylation of a substrate by a multistep enzymatic process that includes an ubiquitin activator (E1), an ubiquitin conjugator (E2), and an ubiquitin ligase (E3). Ubiquitinylated substrates may then be targeted for degradation by the proteasome (Hershko and Ciechanover, 1998). Parkin is an E3 ubiquitin ligase (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000) and mediates the ubiquitinylation of itself (Staropoli et al., 2003; Zhang et al., 2000) and various protein substrates (Chung et al., 2001; Corti et al., 2003; Huynh et al., 2003; Imai et al., 2001; Ren et al., 2003; Shimura et al., 2001; Staropoli et al., 2003; Tsai et al., 2003; Zhang et al., 2000). A subset of mutations associated with autosomal recessive juvenile Parkinson's disease (AR-JPD) results in the loss of parkin E3 function (Imai et al., 2000, 2001; Shimura et al., 2000; Zhang et al., 2000), which likely leads to UPS dysfunction and the accumulation of parkin substrates, resulting in neurodegeneration (Giasson and Lee, 2003). Indeed, overexpression of parkin suppresses neurodegeneration associated with UPS dysfunction (Petrucelli et al., 2002) or endoplasmic reticulum (ER) stress (Imai et al., 2001; Yang et al., 2003), whereas targeted decreases in parkin expression augment cell death (Petrucelli et al., 2002; Yang et al., 2003).

In brain, parkin associates with the molecular chaperone heat shock protein 70 (Hsp70) (Imai et al., 2002), and both Hsp70 (Auluck et al., 2002; McLean et al., 2002) and parkin (Schlossmacher et al., 2002) are found in LBs in sporadic PD. Hsp70 prevents the formation of protein aggregates (Adachi et al., 2003; Chan et al., 2000; Muchowski et al., 2000) and is neuroprotective in both *Drosophila* (Auluck et al., 2002; Chan et al., 2000; Warrick et al., 1999) and mammalian models (Adachi et al., 2003; Cummings et al., 2001) of neurodegenerative disease.

The role of parkin and Hsp70 in sporadic PD is not clearly understood. However, it is possible that loss of parkin (Ardley et al., 2003; Winklhofer et al., 2003) and Hsp70 (Sherman and Goldberg, 2001) function may occur through their sequestration in LBs as a result of UPS dysfunction and cellular stress. It is also possible that negative modulators of parkin and Hsp70 exist and contribute to the pathogenesis of sporadic PD. Indeed, it has been recently shown that S-nitrosylation of parkin

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results in the inhibition of parkin E3 activity both in vitro and in vivo, providing a possible mechanistic link between the genetic and sporadic forms of PD (Chung et al., 2004; Yao et al., 2004).

BAG1, a Hsp70 interacting protein that contains a prototypic BAG domain at its C terminus, was cloned based on its synergistic interaction with *bcl-2* (Takayama et al., 1995) and is generally considered to confer resistance to cell death (Takayama and Reed, 2001). BAG1 functions as a cochaperone of Hsp70 through the interaction of its BAG domain and the ATPase domain of Hsp70 (Briknarova et al., 2001; Hohfeld and Jentsch, 1997; Sondermann et al., 2001; Takayama et al., 1997; Zeiner et al., 1997). There are currently six known human BAG family members (*BAG1–6*), and homologs have been identified in yeast, invertebrates, mammals, and plants (Takayama and Reed, 2001). BAG5 is the only BAG family member that is predicted to contain multiple BAG domains and currently remains uncharacterized. Aside from the formation of the BAG-Hsp70 complex, BAG proteins functionally interact with a variety of binding partners and coordinate diverse cellular processes such as stress signaling (Song et al., 2001), cell division, cell death, and cell differentiation (Takayama and Reed, 2001).

Here, we show that BAG5, a BAG domain-containing family member, interacts with both Hsp70 and parkin with deleterious functional consequences. Through these interactions, BAG5 inhibits Hsp70 chaperone activity and parkin E3 ubiquitin ligase activity and enhances the sequestration of parkin within LB-like protein aggregates. In contrast, short hairpin-mediated depression of BAG5 expression decreases the sequestration of parkin within protein aggregates. Furthermore, BAG5 also mitigates parkin-mediated preservation of proteasome function and inhibition of cell death. Finally, we show that targeted expression of BAG5 in an in vivo model of PD enhances dopaminergic neurodegeneration, whereas a mutant form of BAG5 that can inhibit BAG5 function results in enhanced cell survival.

Results

BAG5 Expression Is Induced in Dopaminergic Neurons after Injury

To identify molecular mediators of dopaminergic neurodegeneration, we employed differential display analysis following the unilateral transection of the axons of dopaminergic SNpc neurons within their projection pathway, the medial forebrain bundle (MFB), in rat. The partial cDNA of a transcript that increased in the SNpc when compared to the contralateral untransected side at 24 hr postaxotomy (see the Supplemental Data at http://www.neuron.org/cgi/content/full/44/6/931/DC1/) was expanded by RT-PCR and RACE. The full-length cDNA encoded for a 447 amino acid protein with high identity to human *BAG5* (90.4% identity; GenBank accession number NM_004873) and mouse *BAG5* (*bcl-2-associated athanogene 5*) (93.7% identity; GenBank accession number XM_127149) (Figure 1A).

Using species-specific probes directed against the open reading frame of *BAG5*, we identified two transcripts of *BAG5* that were widely and differentially ex-

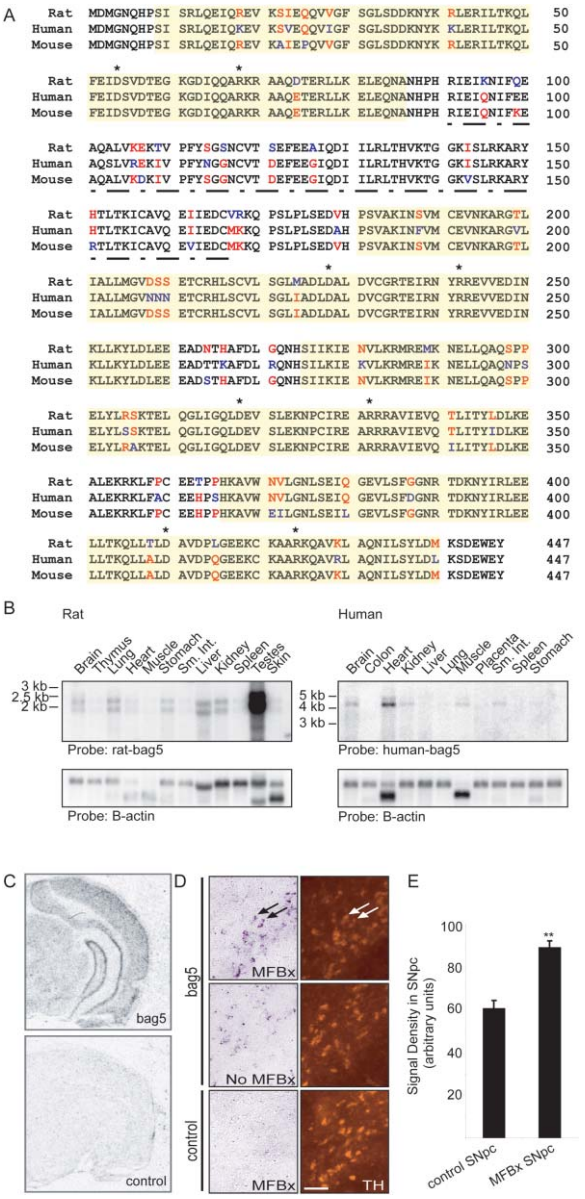

Figure 1. Cloning and Characterization of BAG5

(A) Sequence of cloned rat BAG5 aligned with human and mouse sequences. The four BAG domains are shaded, and a putative "short" fifth BAG domain (Briknarova et al., 2002) is underlined. Substituted residues in BAG5(DARA) are indicated with an asterisk.

(B) Northern blots of BAG5 expression in rat and human.

(C) In situ hybridization (ISH) of adult rat brain using a BAG5-specific antisense radiolabeled probe. The lower panel is a representative ISH using a control sense probe.

(D) Combined ISH and TH immunohistochemistry (IHC) in SNpc of rat brain using a DIG-BAG5 probe shows that TH^+ neurons are also $BAG5\text{-}mRNA^+$ (arrows, upper panels). The lower panels show a representative combined ISH and IHC using a control sense BAG5 probe. The upper and middle panels are from the SNpc ipsilateral and contralateral to MFB axotomy (MFBx), respectively.

(E) Quantitative analysis of ISH signal density in the SNpc at 24 hr postaxotomy (mean ± SEM; $^{**}p < 0.001$; paired Student's t test; $n = 4$; three sections per animal).

pressed in multiple tissue types, including rat and human brain (Figure 1B). In situ hybridization (ISH) analysis in rat brain using an antisense cRNA probe directed against rat BAG5 revealed BAG5 expression in cortex, hippocampus, red nucleus, and the SNpc (Figure 1C, upper panel). A control sense cRNA probe did not reveal any structure-specific signal above background (Figure 1C, lower panel). ISH with digoxygenin (DIG)-labeled antisense rat BAG5 probes combined with immunohistochemistry (IHC) for tyrosine hydroxylase (TH), a marker for dopaminergic neurons, demonstrated that BAG5 mRNA was expressed in the dopaminergic neurons of the SNpc (Figure 1D). We also verified the increase in BAG5 expression in these neurons at 24 hr following MFB axotomy both qualitatively (Figure 1D, upper panel versus middle panel) and quantitatively (Figure 1E).

We used polyclonal antibodies directed against BAG5 to examine BAG5 protein expression. The specificity of the BAG5 antibody was confirmed by Western blot analysis of cell lysates from the dopaminergic cell line SH-SY5Y transfected with N-terminal FLAG epitope-tagged BAG5 (FLAG-BAG5) (Figure 2A). Both anti-FLAG and anti-BAG5 antibodies recognized a band with the predicted molecular weight. Preabsorption of the anti-BAG5 antibody with its corresponding peptide immunogen competed out the signal. Probing lysates from mouse and human brain revealed a band of the expected molecular weight of approximately 51 kDa (Figure 2B, upper panel); preincubation of the anti-BAG5 antibody with its corresponding peptide immunogen also competed out the signal as seen by Western blot (Figure 2B, lower panel) and immunohistochemistry (Figure 2C). The anti-BAG5 antibody also recognized rat BAG5, albeit with decreased sensitivity and occasionally a minor band of approximately 35 kDa in BAG5-transfected cell lysates and tissue lysates (data not shown). In mouse and rat SNpc, BAG5 immunoreactivity was found in all TH-immunopositive (TH^+) dopaminergic neurons, as well as some TH-immunonegative (TH^-) neurons (Figures 2D and 2E). Similarly, in human SNpc from a patient with diffuse Lewy body (DLB) disease, a neurodegenerative disorder similar to PD, BAG5 immunoreactivity was found in TH^+ neurons (Figure 2F). α-synuclein is a major component of LBs (Spillantini et al., 1997), and we found BAG5 immunoreactivity in α-synuclein immunoreactive neurons (Figure 2G) and localized within all LBs (Figure 2H). Our finding that BAG5 expression is induced following dopaminergic neuron injury and is localized within dopaminergic neurons and LBs led us to hypothesize that BAG5 may play a role in neurodegeneration.

BAG5 Interacts with and Inhibits the Molecular Chaperone Hsp70

Since other BAG family proteins interact with the chaperone Hsp70 (Briknarova et al., 2001; Hohfeld and Jentsch, 1997; Sondermann et al., 2001; Takayama et al., 1997; Zeiner et al., 1997), a protein known to be present in LBs (Auluck et al., 2002; McLean et al., 2002) and to prevent both protein aggregation and neurodegeneration (Adachi et al., 2003; Auluck et al., 2002; Cummings et al., 2001; Sherman and Goldberg, 2001), we tested whether BAG5 also interacts directly with Hsp70. To test this, we performed in vitro GST pull-down assays (PDAs) using recombinant fusion proteins.

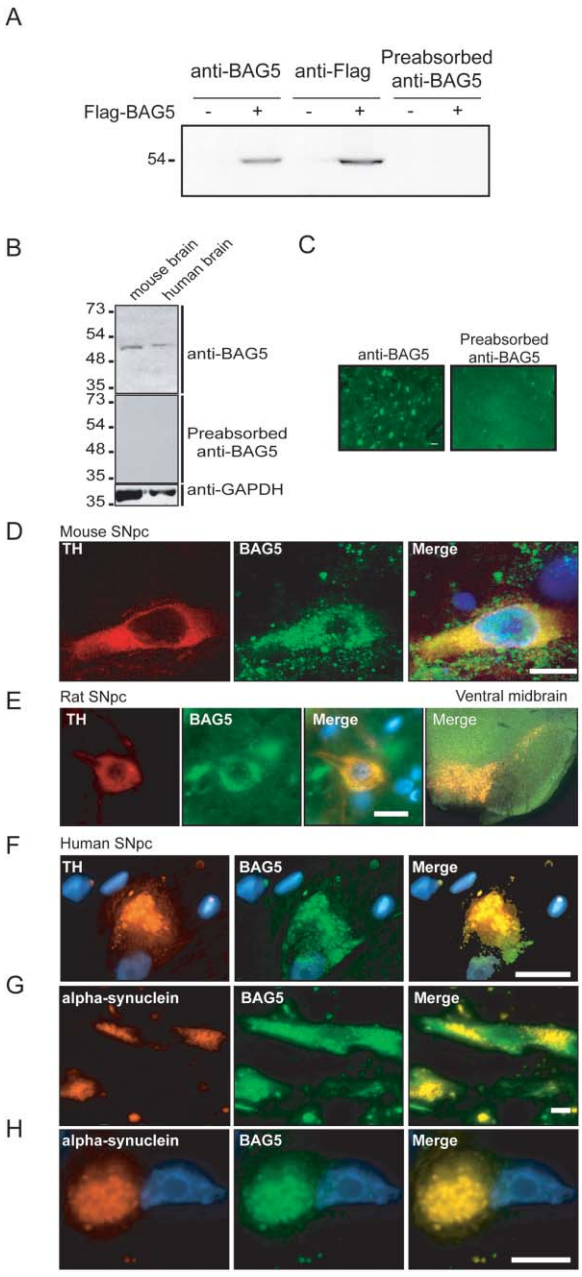

Figure 2. BAG5 Protein Is Expressed in the SNpc of Rodent and Human Brain

(A) Western blot (WB) of transfected SH-SY5Y lysate (control [−] or pDEST-FLAG-BAG5 vector [+]) probed with anti-BAG5 and anti-FLAG. Preabsorption of anti-BAG5 antibody with BAG5 peptide immunogen is shown on the right. (B) WB of mouse brain and human brain lysates using the anti-BAG5 antibody. (C) IHC of human cortex with anti-BAG5 (left panel) or preabsorbed anti-BAG5 (right panel). IHC of mouse (D) and rat (E) SNpc shows BAG5 immunoreactivity (green) colocalized with TH^+ neurons (red). BAG5 immunoreactivity colocalizes with TH immunoreactivity throughout the SNpc and ventral tegmental area (VTA) of midbrain ([E], right panel). (F) BAG5 immunoreactivity (green) is colocalized in TH^+ neurons (red) in the SNpc from a patient with DLB. (G) BAG5 immunoreactivity (green) colocalizes in α-synuclein$^+$ neurons (red) in human DLB SNpc. (H) BAG5 immunoreactivity is also present in α-synuclein$^+$ perinuclear Lewy bodies. Scale bars, 50 μm (C), 10 μm (D and E), and 20 μm (F–H). Blue staining in (D)–(H) is the nuclear stain Hoechst 33342. Molecular weight markers are shown in kDa.

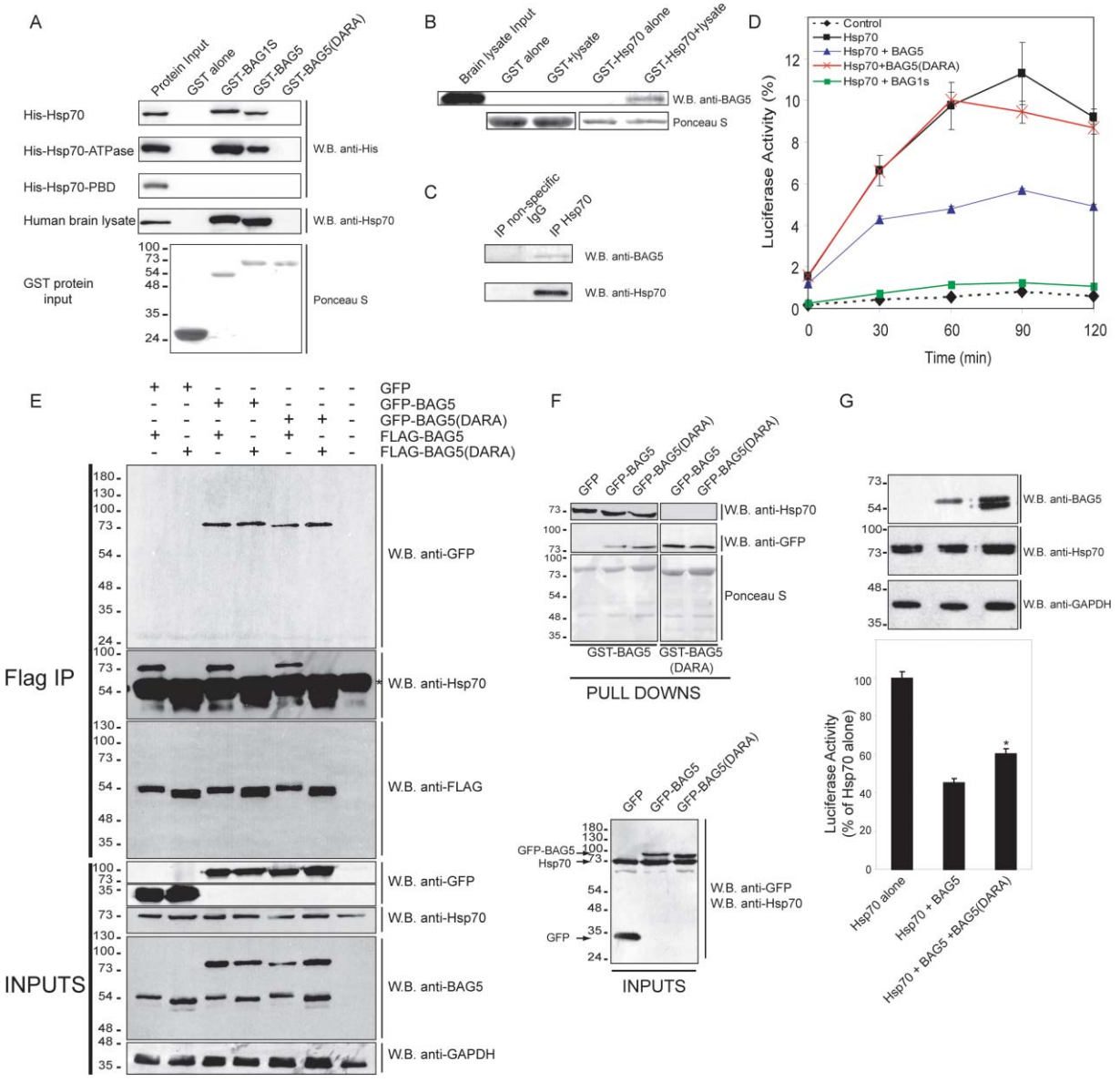

Figure 3. BAG5 Is a Cochaperone of Hsp70

(A) GST-BAG5 pull-down assay with His-Hsp70, His-Hsp70 ATPase domain, His-Hsp70 PBD, or human brain SNpc lysate (400 μg). (B) GST-Hsp70 pull-down assay from human brain SNpc lysate shows Hsp70 binding to endogenous BAG5. (C) Coimmunoprecipitation of endogenous Hsp70 and BAG5 from human brain lysate (2 mg). (D) Luciferase refolding assay in HEK293T cells. (E) BAG5 associates with Hsp70 and itself as shown by coimmunoprecipitation of GFP-BAG5 and FLAG-BAG5 from transfected HEK293T lysate. The asterisk indicates the immunoglobulin heavy chain. (F) GST-BAG5 and GST-BAG5(DARA) pull-down assays from HEK293T cell lysates transfected with GFP, GFP-BAG5, or GFP-BAG5(DARA). Inputs are shown in the lower Western blot (WB). (G) Luciferase refolding assay in HEK293T cells shows BAG5(DARA) mitigates BAG5 inhibition of Hsp70-mediated refolding of thermally inactivated luciferase (*$p < 0.05$; Student's t test). Representative WBs are shown above the graph. For (A)–(G), representative results from three experiments are shown.

We found that GST-BAG5, like GST-BAG1, pulled down full-length Hsp70 in vitro, whereas GST-alone did not (Figure 3A). To identify the region of Hsp70 that mediates the interaction with BAG5, we generated His-Hsp70 deletion constructs containing only the ATPase domain (ATPase) or protein binding domain (PBD) of Hsp70. Both GST-BAG5 and GST-BAG1, but not GST-alone, pulled down the Hsp70 ATPase domain in vitro. In contrast, GST-BAG5, GST-BAG1, and GST-alone did not pull down the Hsp70 PBD (Figure 3A). Therefore, the ATPase domain of Hsp70 is both necessary and sufficient for the interaction between BAG5 and Hsp70.

To further confirm the specificity of the interaction between BAG5 and Hsp70, we aligned its BAG domains with the BAG domain of BAG1 to identify conserved amino acids. Based on our alignment and the crystal structure of the BAG domain of BAG1 bound to the Hsp70 ATPase domain (Sondermann et al., 2001), we substituted the conserved aspartate (D) and arginine (R) with alanine (A) in all four predicted BAG domains by

site-directed mutagenesis to generate the mutant BAG5(DARA) (Figure 1A). Consistent with predictions from previous structural studies (Briknarova et al., 2001, 2002; Sondermann et al., 2001), substitution of these conserved residues in all four BAG domains abolished the interaction with full-length Hsp70 and the Hsp70 ATPase domain in PDAs using GST-BAG5(DARA). Furthermore, we found that GST-BAG5 associated with endogenous Hsp70 in human brain lysate, while GST alone or the mutant GST-BAG5(DARA) did not (Figure 3A). In the converse experiment, the GST-Hsp70 fusion protein also associated with endogenous BAG5 (Figure 3B). We confirmed the physical association between BAG5 and Hsp70 in vivo by coimmunoprecipitation from human brain lysate (Figure 3C). Thus, BAG5 interacts with Hsp70 in vitro and in vivo, and substitution of select residues within the BAG domains is sufficient to abolish this interaction.

We next examined the functional consequence of the interaction between Hsp70 and BAG5. Other BAG family proteins, including BAG1, are cochaperones of Hsp70 and can negatively regulate the ability of Hsp70 to refold misfolded proteins both in vitro (Hohfeld and Jentsch, 1997; Takayama et al., 1997; Zeiner et al., 1997) and in vivo (Nollen et al., 2001). Therefore, we hypothesized that BAG5 could also modulate Hsp70 chaperone function. Using a previously described system to investigate the effect of BAG1 on Hsp70 chaperone activity in cells (Nollen et al., 2000, 2001), we examined the effect of BAG5 on Hsp70 activity. We transiently cotransfected Hsp70 and a luciferase reporter into HEK293T cells with BAG1, BAG5, or the mutant BAG5(DARA). Luciferase activity was thermally inactivated, and its reactivation by Hsp70 was measured at 30 min intervals over 2 hr. By 2 hr postinactivation, BAG5 and BAG1 significantly diminished Hsp70-mediated luciferase reactivation, whereas the mutant BAG5(DARA) had no significant effect (Figure 3D). These results show that BAG5 negatively regulates the chaperone activity of Hsp70.

BAG5 Associates with Itself and with the Mutant BAG5(DARA)

Takayama et al. (1997) have suggested a minimal heterotetramer stoichiometry (2:2) for the BAG1-Hsp70 interaction and have confirmed dimer formation by BAG1 alone. Therefore, we tested whether BAG5 may associate with itself by cotransfecting N-terminal GFP-tagged BAG5 (GFP-BAG5) with FLAG-BAG5 in HEK293T cells. Coimmunoprecipitation of GFP-BAG5 but not GFP alone with FLAG-BAG5 suggested that BAG5 can associate with itself (Figure 3E, lane 3). Furthermore, GFP-BAG5 (DARA) also coimmunoprecipitated with FLAG-BAG5, suggesting that site-directed substitution within the BAG domains does not significantly alter the conformation of the mutant BAG5(DARA) (Figure 3E, lane 5). Indeed, both GFP-BAG5 and GFP-BAG5(DARA) coimmunoprecipitated with FLAG-BAG5(DARA) (Figure 3E, lanes 4 and 6, respectively). We also confirmed that Hsp70 coimmunoprecipitated with FLAG-BAG5 and not FLAG-BAG5(DARA) (Figure 3E, lanes 1, 3, and 5 versus 2, 4, and 6). We further demonstrated the interaction between BAG5 and itself, and BAG5 and the mutant BAG5(DARA), using PDAs in cell lysates transfected with GFP-BAG5 and GFP-BAG5(DARA). Similar to results from Figure 3E, GST-BAG5 and GST-BAG5(DARA) interacted with both GFP-BAG5 and GFP-BAG5(DARA), whereas only GST-BAG5 interacted with Hsp70 (Figure 3F). To determine if this association has a functional consequence, we cotransfected BAG5 and BAG5(DARA) together in the luciferase refolding assay described above and found that the mutant BAG5(DARA) inhibits the effect of BAG5 on Hsp70-mediated refolding of the luciferase (Figure 3G). Therefore, complexes of BAG5 that contain BAG5(DARA) are unable to bind Hsp70 and may inhibit BAG5 activity.

BAG5 Interacts Directly with Parkin

In brain, Hsp70 associates with parkin (Imai et al., 2002). Because of the importance of parkin in the pathogenesis of PD and its association with Hsp70, we tested whether BAG5 associates in a complex with Hsp70 and parkin. Thus, we cotransfected GFP-parkin with FLAG-BAG5 or FLAG-BAG5(DARA) and His-Hsp70 in HEK293T cells. Immunoprecipitation (IP) of GFP-parkin resulted in the coimmunoprecipitation of both Hsp70 and BAG5 or BAG5(DARA) (Figure 4A). Furthermore, IP of GFP-parkin resulted in the coimmunoprecipitation of BAG5 or BAG5 (DARA) in the absence of overexpressed Hsp70.

To further examine the nature of the association between parkin, Hsp70, and BAG5, we performed in vitro pull-down experiments using GST-Hsp70, GST-BAG5, or GST-BAG5(DARA) with recombinant N-terminal His-tagged parkin. GST-Hsp70, GST-BAG5, and GST-BAG5 (DARA) all interacted directly with His-parkin, whereas GST-alone did not (Figure 4B). Thus, BAG5 can interact directly with parkin in the absence of Hsp70.

We sought to further dissect the interaction between BAG5 and parkin using a series of N-terminal GFP-tagged parkin deletion constructs (Figure 4C). The parkin deletion constructs were individually transfected in HEK293T cells, and PDAs were performed using GST-BAG5. BAG5 associated with both the N-terminal and C-terminal deletion constructs of parkin. Within the N terminus of parkin, the BAG5 interaction was further mapped exclusively to the linker region of parkin (Figure 4D). In agreement with previous results (Imai et al., 2002; Tsai et al., 2003), Hsp70 binding was limited to the C terminus of parkin (data not shown). Taken together with the findings from the coimmunoprecipitation experiments and in vitro PDAs described above, these results establish that BAG5, Hsp70, and parkin may associate in a complex. Furthermore, BAG5 can interact directly with parkin in the absence of Hsp70.

BAG5 Inhibits Parkin E3 Activity

Parkin, like other E3s, can undergo autoubiquitinylation (Chung et al., 2004; Staropoli et al., 2003; Zhang et al., 2000), and parkin E3 activity may be modulated by Hsp70 (Imai et al., 2002; Tsai et al., 2003). To investigate the potential role of BAG5 on parkin E3 activity, we examined the autoubiquitinylation of parkin in the presence or absence of BAG5 and/or Hsp70 in vitro. GST-parkin but not GST-alone underwent in vitro autoubiquitinylation in the presence of E1 and the E2s Ubc6ΔC and Ubc7 (Imai et al., 2001) as detected by anti-ubiquitin antibodies (Figure 4E). The removal of either ubiquitin

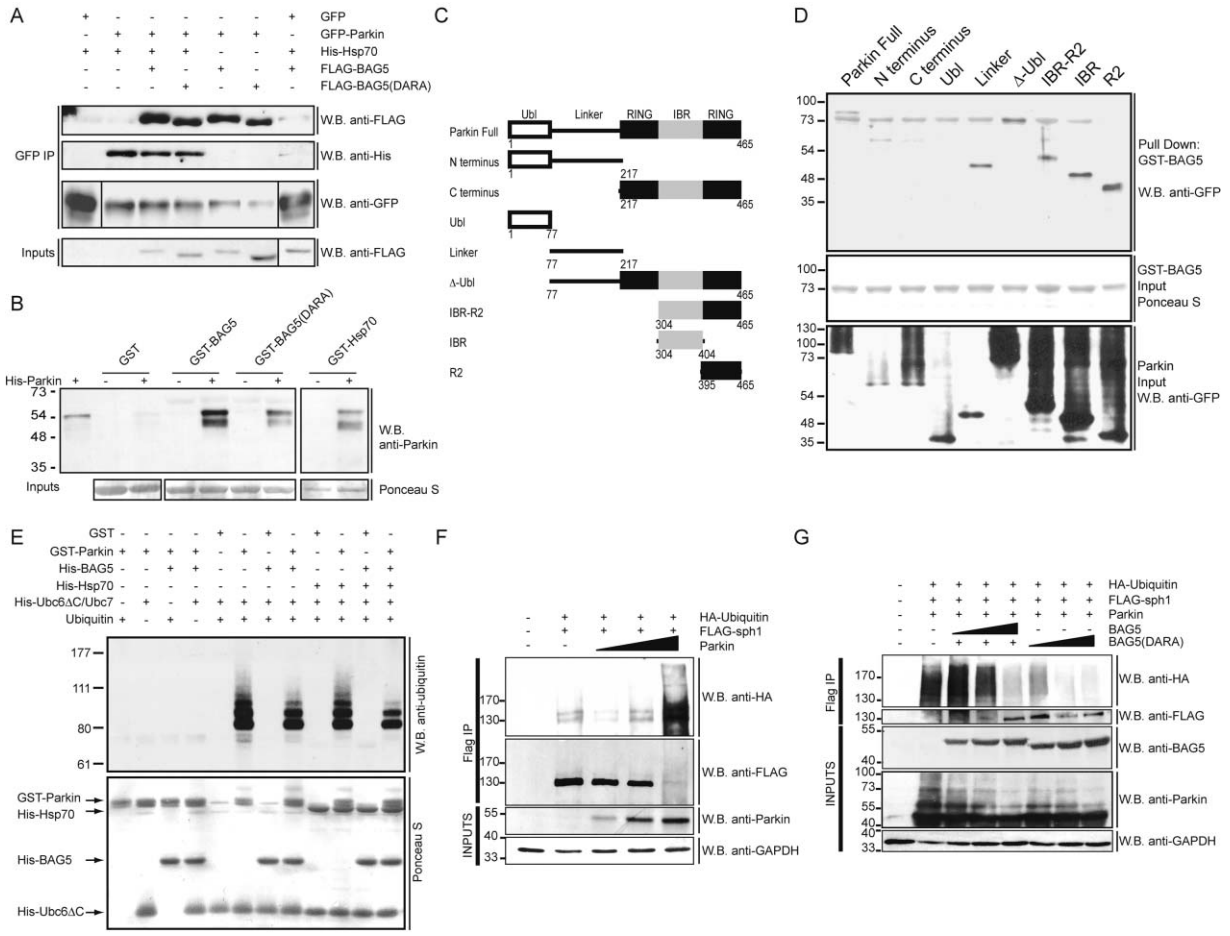

Figure 4. BAG5 Interacts Directly with Parkin and Inhibits Parkin E3 Activity

(A) BAG5 associates with both parkin and Hsp70 as shown by coimmunoprecipitation from lysates of transfected HEK293T cells. (B) GST pull-down assay demonstrating that BAG5, BAG5(DARA), and Hsp70 directly interact with His-parkin. (C) Schematic of parkin deletion constructs. (D) GST-BAG5 pull-down assays from HEK293T cell lysate transfected with indicated GFP-parkin deletion constructs. (E) In vitro ubiquitinylation assay shows that BAG5 inhibits parkin autoubiquitinylation. (F) Ubiquitinylation assay in transfected HEK293T cells shows that parkin enhances the ubiquitinylation of synphilin (FLAG-sph1). (G) Ubiquitinylation assays in transfected HEK293T cells show that both BAG5 and BAG5(DARA) inhibit parkin-mediated ubiquitinylation of the substrate synphilin. (A)–(G) are representative results of three independent experiments.

or the E2s Ubc6ΔC and Ubc7 prevented autoubiquitinylation of parkin. The addition of His-Hsp70 alone did not affect autoubiquitinylation of parkin, whereas the addition of His-BAG5 alone significantly inhibited autoubiquitinylation. Furthermore, the addition of Hsp70 was unable to rescue the BAG5-mediated inhibition of autoubiquitinylation of parkin (Figure 4E). Parkin ubiquitinylation was confirmed by reprobing the blot with an anti-parkin antibody (data not shown). The integrity and equivalent loading of all fusion proteins were verified by Ponceau S staining (Figure 4E). Taken together, these data demonstrate that BAG5 can directly inhibit parkin-mediated autoubiquitinylation independently of Hsp70.

Parkin has been shown to ubiquitinylate several substrates including the α-synuclein interacting protein synphilin (Chung et al., 2001, 2004). We also observed an enhancement in the ubiquitinylation of C-terminal FLAG-tagged synphilin (FLAG-sph1) when cotransfected with parkin in HEK293T cells treated with the proteasome inhibitor MG132 (Figure 4F). A BAG5 dose-dependent decrease in the amount of immunoprecipitated ubiquitinylated synphilin was observed (Figure 4G). Similarly, we tested the mutant BAG5(DARA), which retains the ability to directly interact with parkin, and found that it could also significantly inhibit parkin-mediated ubiquitinylation of synphilin (Figure 4G). Therefore, BAG5 is a putative inhibitor of parkin E3 activity.

BAG5 Promotes Parkin Sequestration through the Inhibition of Hsp70

Proteasomal inhibition mediates the sequestration of parkin within perinuclear protein aggregates similar to the LBs of PD (Ardley et al., 2003; Junn et al., 2002; Muqit et al., 2003; Tsai et al., 2003; Winklhofer et al., 2003). In experiments similar to that of Junn et al. (2002), we transfected HEK293T cells with GFP-parkin and treated the cells with MG132 to initiate the formation of protein aggregates (Figure 5A). We confirmed that the perinuclear parkin protein aggregates were immunoreactive for ubiquitin, and we found that Hsp70 immunore-

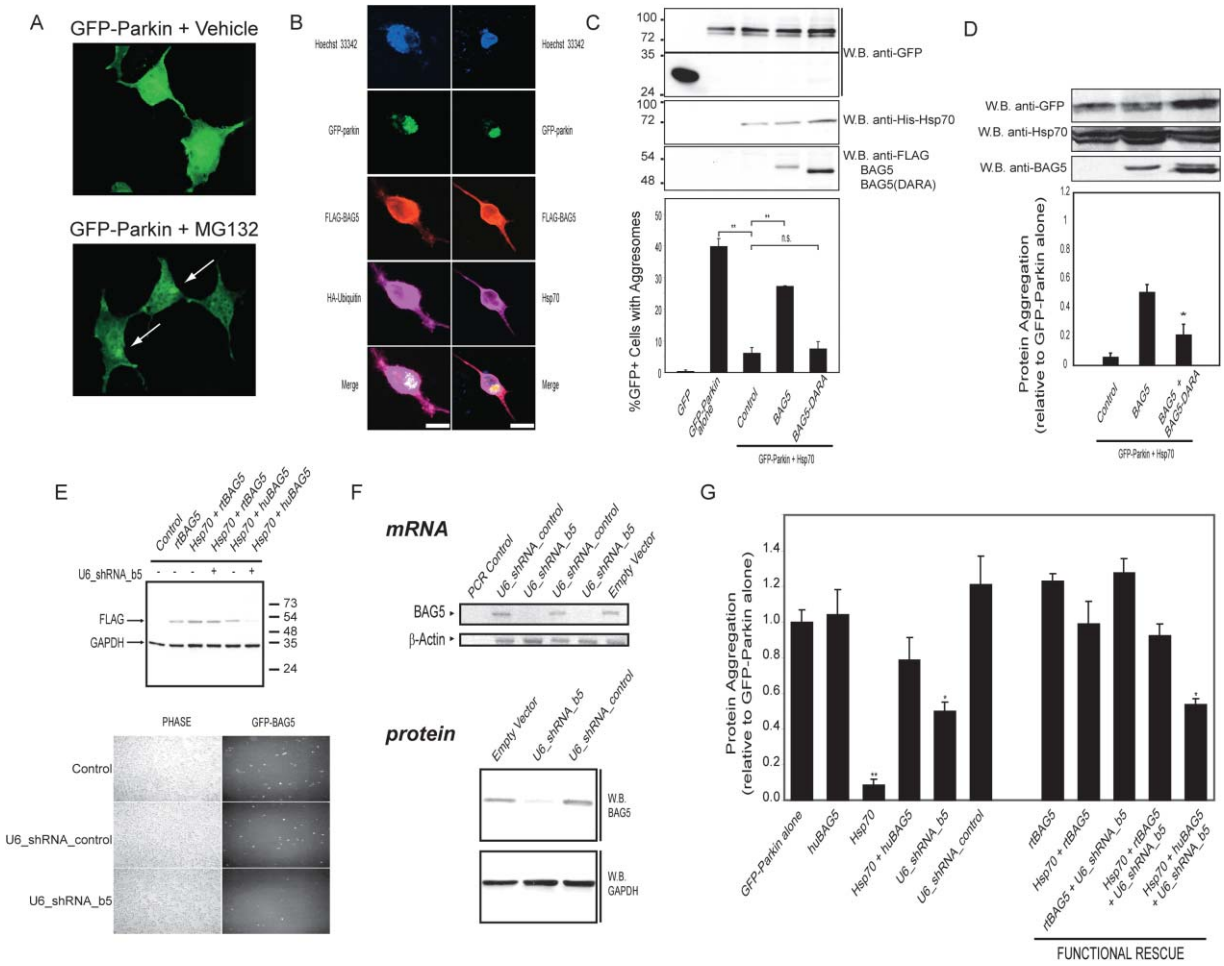

Figure 5. BAG5 Prevents Hsp70-Mediated Inhibition of the Formation of Parkin-Containing Protein Aggregates

(A) Parkin is sequestered in protein aggregates following proteasome inhibition by MG132 in HEK293T cells (arrows). (B) Parkin-containing protein aggregates are also immunoreactive for BAG5 and ubiquitin (left panels) and are surrounded by immunoreactivity for Hsp70 (right panels). Scale bars, 10 μm. (C) BAG5 inhibits Hsp70-mediated prevention of parkin aggregation, whereas BAG5(DARA) does not. (D) The mutant BAG5(DARA) mitigates BAG5-mediated inhibition of Hsp70 in the parkin aggregation assay. Corresponding representative WBs are shown above the graphs in (C) and (D). (E) U6_shRNA_b5 depresses FLAG-huBAG5 protein expression but is unable to depress FLAG-rtBAG5 expression as shown by WBs. Lower panels show that U6_shRNA_b5 depresses GFP-BAG5 expression in HEK293T cells, whereas empty vector (Control) and an unrelated shRNA (U6_shRNA_control) do not. (F) U6_shRNA_b5 but not U6_shRNA_control depresses endogenous BAG5 mRNA and protein expression in HEK293T cells as shown by RT-PCR and WB. (G) U6_shRNA_b5-mediated depression of BAG5 significantly decreases the relative sequestration of parkin in protein aggregates, whereas U6_shRNA_control does not. Protein aggregation in the graph is relative to aggregation of GFP-parkin alone. (C)–(G) are representative results of at least three independent experiments; for graphs, mean ± SEM; *$p < 0.05$; **$p < 0.001$ (Student's t test; Bonferroni-corrected; n.s., not significant).

activity was localized mainly at the periphery of the aggregate (Figure 5B) as previously described (Junn et al., 2002). We found that BAG5 immunoreactivity was also colocalized within these LB-like aggregates (Figure 5B) as was α-synuclein and synphilin (data not shown). Furthermore, in agreement with previous studies (Ardley et al., 2003; Junn et al., 2002), 40% ± 3% of GFP-parkin$^+$ cells contained aggregates after MG132 treatment. To verify that the sequestration of parkin within protein aggregates was specific to parkin, we transfected cells with GFP alone and found that less than 1% of cells contained any detectable inclusions (Figure 5C).

The solubility of parkin and its mutants within a cell can be enhanced by the coexpression of the chaperones Hsp70 and Hsp40 with parkin (Winklhofer et al., 2003). Thus, we examined whether Hsp70 and BAG5 had an effect on the formation of parkin-containing perinuclear aggregates. The cotransfection of BAG5 or BAG5(DARA) alone with GFP-parkin did not influence the formation of parkin-containing protein aggregates. Cotransfection of Hsp70 with GFP-parkin prior to proteasome inhibition significantly reduced the sequestration of parkin within aggregates to only 6% ± 2% of GFP-parkin$^+$ cells ($p < 0.001$; Student's t test). However, BAG5 significantly inhibited the ability of Hsp70 to prevent aggregation, whereas cotransfection of the mutant BAG5(DARA) did not (Figure 5C). Given that the mutant BAG5(DARA) inhibited the effect of BAG5 on Hsp70 (Figure 3G), we tested whether the coexpression of BAG5(DARA) could also mitigate BAG5 inhibition of Hsp70-mediated sup-

pression of parkin sequestration. Indeed, we found a significant decrease in the number of GFP-parkin-containing protein aggregates when BAG5 was cotransfected with BAG5(DARA) (Figure 5D). The expression levels of GFP-parkin were similar in all conditions tested. Taken together, these data suggest that BAG5, through its inhibition of Hsp70, can promote parkin sequestration and enhance the formation of LB-like protein aggregates.

shRNA-Mediated Knockdown of BAG5 Inhibits the Sequestration of Parkin

To further validate the functional role of BAG5 as an enhancer of parkin sequestration in protein aggregates, we employed short hairpin RNA (shRNA)-mediated "knockdown" (Paddison et al., 2002) of BAG5 and examined its effect on the sequestration of parkin. U6_shRNA_b5, a U6 promoter-driven shRNA directed against human BAG5, was able to significantly depress the expression of transfected FLAG-tagged human BAG5 in HEK293T cells, whereas a U6_shRNA_control vector targeting luciferase could not (Figure 5E, upper panel). Also, cotransfection of U6_shRNA_b5 with N-terminal GFP-tagged BAG5 in HEK293T cells depressed the expression of GFP-BAG5 protein, whereas U6_shRNA_control had no effect on GFP-BAG5 (Figure 5E, lower panels). Next, we tested if U6_shRNA_b5 could depress the endogenous huBAG5 in cells, and we found that U6_shRNA_b5 significantly depressed the relative expression of both endogenous BAG5 mRNA and protein, whereas U6_shRNA_control did not (Figure 5F). We then examined the relative effect of shRNA-mediated BAG5 knockdown on the sequestration of parkin in protein aggregates. Cotransfection of U6_shRNA_b5 with GFP-parkin significantly inhibited the sequestration of parkin in aggregates to 50% $\pm$ 5% relative to GFP-parkin alone, whereas U6_shRNA_control had no significant effect (Figure 5G).

To confirm that our observed knockdown effect was due to the specific depression of BAG5, we performed functional rescue experiments with rtBAG5, which is highly homologous to huBAG5 but resistant to U6_shRNA_b5-mediated knockdown (Figure 5E). Since BAG5 may promote parkin sequestration through its inhibition of Hsp70 (Figure 5C), we first examined if rtBAG5 also mitigated Hsp70-mediated inhibition of parkin sequestration in protein aggregates. Indeed, coexpression of rtBAG5 with Hsp70 and parkin prevented Hsp70-mediated inhibition of parkin sequestration. Significantly, rtBAG5 was able to functionally rescue the loss of huBAG5 inhibition of Hsp70 function in the presence of U6_shRNA_b5 (Figure 5G). Taken together, these data confirm that BAG5 has a functional role in the sequestration of parkin in protein aggregates.

BAG5 Inhibits Parkin-Mediated Suppression of UPS Dysfunction and Cell Death

A GFPu reporter construct (Bence et al., 2001) allows for the measurement of proteasomal activity in living cells. GFPu has been shown to rapidly accumulate in settings of UPS dysfunction due to the expression of mutant proteins associated with neurodegenerative diseases (Bence et al., 2001; Petrucelli et al., 2002; Tsai et al., 2003). Upon cotransfection of synphilin with GFPu in HEK293T cells, we observed a significant accumulation and aggregation of GFPu relative to those cells cotransfected with empty vector (Figures 6A and 6B). To ensure similar transfection efficiency between conditions, we used DsRed and β-galactosidase as transfection controls. Taken together, our results suggest that the accumulation of synphilin alone is sufficient to induce proteasome inhibition as measured by the accumulation of GFPu.

The GFPu reporter is not a substrate of parkin, and parkin has been shown to reduce proteasome impairment due to substrate accumulation as measured by the GFPu reporter system (Petrucelli et al., 2002; Tsai et al., 2003). Similarly, we found that parkin mediated a significant decrease in GFPu accumulation due to synphilin expression (Figure 6C). BAG5 reversed the inhibition of proteasome dysfunction by parkin and resulted in a significant increase in the accumulation of GFPu, whereas cotransfection of the mutant BAG5(DARA) or the chaperone Hsp70 resulted in a significant decrease in the accumulation of GFPu due to synphilin. Overexpression of BAG5 alone also resulted in a significant increase in the accumulation of GFPu due to synphilin expression (data not shown). Furthermore, cotransfection of the inhibitory mutant BAG5(DARA) with BAG5 mitigated proteasome dysfunction as measured by GFPu accumulation (Figure 6D). Taken together, our data suggest that BAG5 may contribute in part to proteasome impairment, through the inhibition of parkin.

Parkin has also been shown to prevent cell death both in cell lines and in vivo (Imai et al., 2000, 2001; Kim et al., 2003; Oluwatosin-Chigbu et al., 2003; Petrucelli et al., 2002; Yang et al., 2003). Therefore, we sought to determine whether the interaction between parkin and BAG5 has consequences on cell survival. As previously reported by others (Chung et al., 2004; Ihara et al., 2003), coexpression of synphilin and wild-type α-synuclein in SH-SY5Y cells in the presence of the irreversible proteasome inhibitor lactacystin results in increased cell death, which can be suppressed by parkin (Figure 6E, left panel). The protective effect of parkin was significantly reduced by coexpression of BAG5 (Figure 6E, left panel), while the coexpression of BAG5 alone with synphilin and α-synuclein expression in the presence of lactacystin did not significantly enhance cell death (Figure 6E, right panel). To further validate our results, we used our U6_shRNA_b5 construct to depress the expression of BAG5 and found a significant decrease in cell death, whereas expression of a U6_shRNA_control construct had no effect (Figure 6E, middle panel). The specificity of this effect was demonstrated with functional rescue by coexpressing rat BAG5 with the U6_shRNA_b5 construct. Finally, the mutant BAG5(DARA) was found to suppress both the inhibitory effect of BAG5 on parkin (Figure 6E, left panel) and when expressed alone (Figure 6E, right panel).

BAG5 Enhances Dopaminergic Neurodegeneration In Vivo

Our data implicate BAG5 as a negative modulator of parkin and Hsp70, two molecules with known neuroprotective properties. Because we cloned BAG5 as an upregulated differentially expressed transcript in the rat MFB axotomy model of dopaminergic neuron injury, we

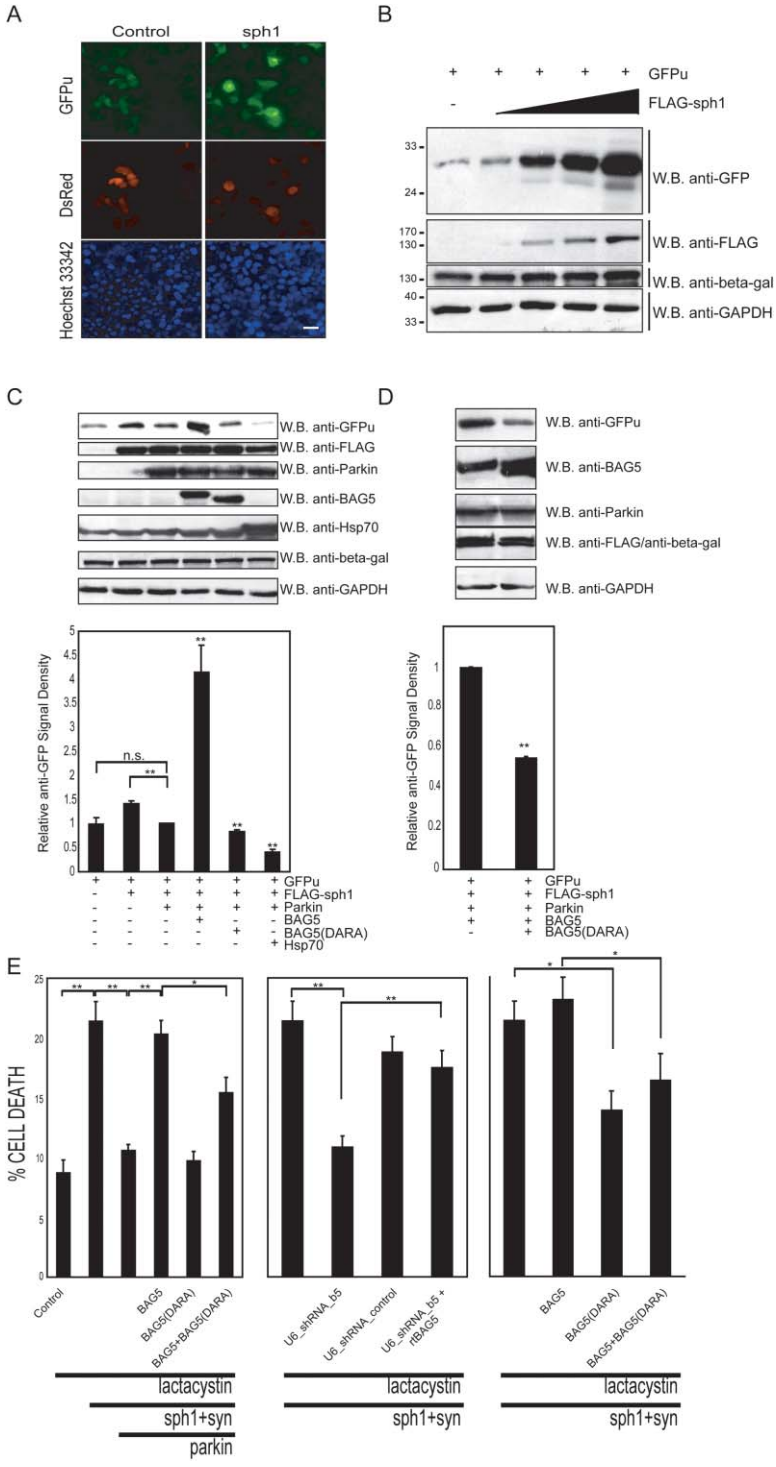

Figure 6. BAG5 Mitigates Parkin-Mediated Inhibition of Proteasome Stress and Cell Death

(A) Synphilin (sph1) enhances the accumulation of GFPu (upper panels). DsRed (middle panels) is a transfection control, and Hoechst 33342 (lower panels) shows total cell numbers in the same field. (B) WB shows a synphilin-mediated dose-dependent increase in GFPu accumulation. (C) BAG5 prevents parkin-mediated rescue of GFPu accumulation due to synphilin. (D) BAG5(DARA) mitigates the effect of BAG5 on GFPu accumulation. Graphs in (C) and (D) show β-galactosidase normalized GFPu signal (mean ± SEM; **$p < 0.001$; n.s., not significant; Student's t test) and corresponding WB. Corresponding WBs of each condition are shown above the graph. (E) BAG5 prevents parkin-mediated inhibition of cell death. Graphs show cell death observed in SH-SY5Y cells transfected with sph1 and synuclein (syn) and treated with the irreversible proteasome inhibitor lactacystin (15 μM) (mean ± SEM; *$p < 0.05$; **$p < 0.001$; n.s., not significant; Student's t test; Bonferroni-corrected).

next examined whether BAG5 can enhance cell death in vivo. A FLAG-BAG5-expressing adenovirus (Ad.BAG5), a FLAG-BAG5(DARA)-expressing adenovirus [Ad.BAG5-(DARA)], and a control adenovirus (Ad.GFP) were constructed. The expression of both FLAG-BAG5 and FLAG-BAG5(DARA) was verified by Western blot analysis of infected cell lysates using both anti-FLAG antibodies (Figure 7A) and anti-BAG5 antibodies (data not shown). Targeted overexpression of adenovirus was achieved in the SNpc at the level of the medial terminal nucleus (MTN) in rat following the unilateral injection of virus in the ipsilateral striatum as we have described previously (Crocker et al., 2001; Smith et al., 2003). Viral expression in the SNpc was verified by immunohistochemistry. FLAG-BAG5 or FLAG-BAG5(DARA) immunoreactivity was detected in TH^+ dopaminergic neurons at 4 weeks postinjection (Figure 7B).

To determine whether BAG5 enhances the loss of dopaminergic neurons following injury, we performed MFB axotomy 1 week after injection of Ad.GFP, Ad.BAG5,

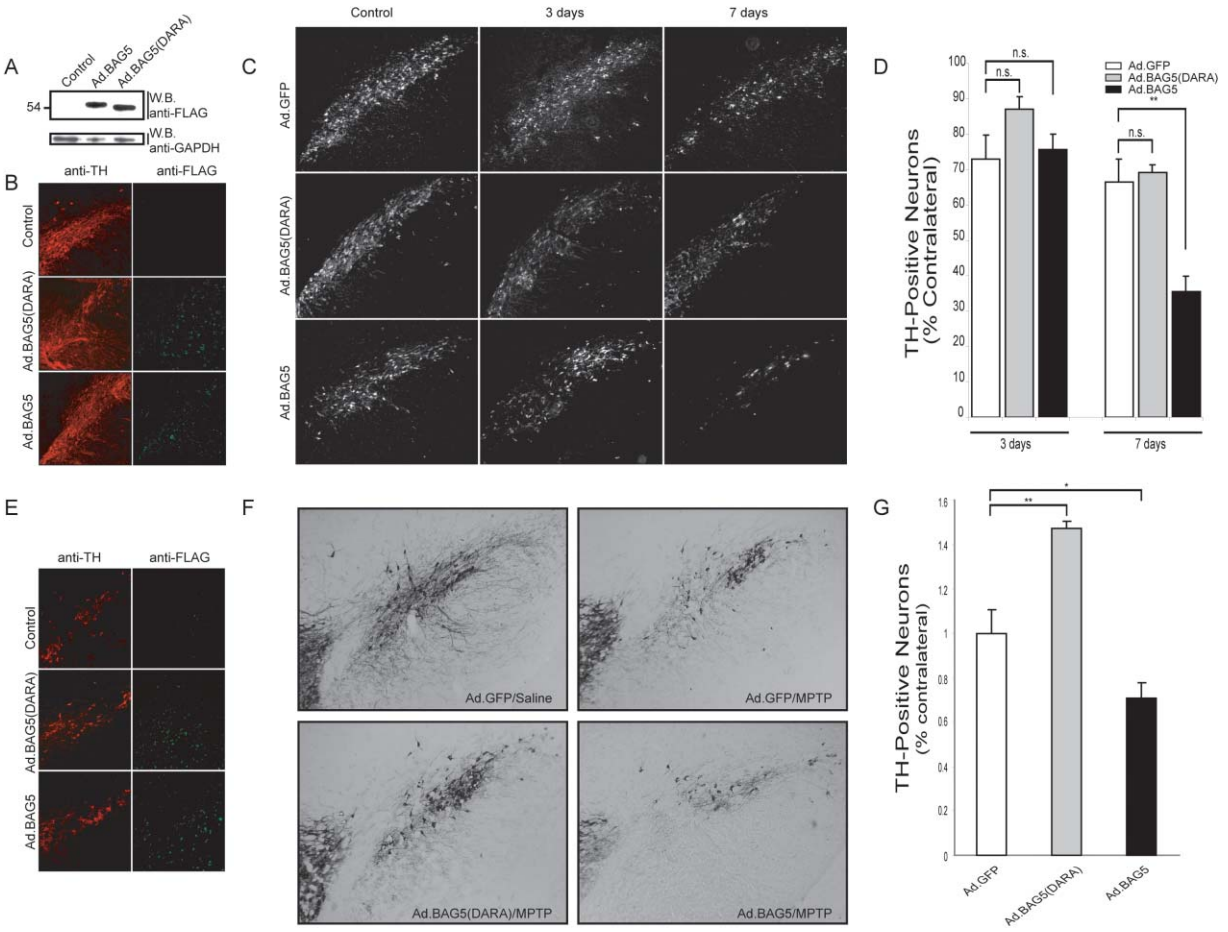

Figure 7. Adenovirus-Mediated Expression of BAG5 in the SNpc Enhances Dopaminergic Neurodegeneration Postaxotomy and in the MPTP Model of PD

(A) WB of lysates of SH-SY5Y infected with Ad.BAG5 and Ad.BAG5(DARA). (B) IHC showing expression of FLAG-BAG5(DARA) and FLAG-BAG5 in the SNpc of rats ipsilateral to the adenovirus-injected striatum and not in the contralateral SNpc (Control). (C) TH immunoreactivity in control SNpc and lesioned SNpc 3 days and 7 days postaxotomy in rats injected with adenovirus. (D) Quantitation of TH-positive neurons as a percent of the contralateral unlesioned. (E) IHC showing expression of adenovirus in the SNpc of mice ipsilateral to the injected striatum and not in the contralateral SNpc (Control). (F) TH immunoreactivity in the SNpc ipsilateral to injection with adenovirus in mice at 2 weeks following treatment with saline or MPTP. (G) Quantitation of TH-positive neurons in the adenovirus-injected SNpc relative to contralateral SNpc. For graphs, mean ± SEM; *$p < 0.05$; **$p < 0.001$ (Student's t test; Bonferroni-corrected; $n = 3–7$).

or Ad.BAG5(DARA) and examined the degree of dopaminergic neuron loss at 3 and 7 days post-MFB axotomy. At day 3 postaxotomy, quantitation of the TH^+ neurons of the treated SNpc relative to the untreated contralateral side revealed no difference in neuronal survival between animals treated with Ad.BAG5, Ad.BAG5(DARA), or Ad.GFP (Figure 7C). However, by day 7 postaxotomy, animals receiving Ad.BAG5 showed increased death of dopaminergic neurons, with only 35% ± 5% of neurons surviving relative to control versus 66% ± 7% (mean ± SEM; $p = 0.001$; Student's t test) of neurons surviving relative to control in animals receiving Ad.GFP (Figures 7C and 7D). Loss of dopaminergic neurons was verified by cresyl violet staining to rule out the possibility that TH protein expression was downregulated postaxotomy (data not shown).

We next examined the effect of targeted expression of BAG5 and the inhibitory mutant BAG5(DARA) in the SNpc in the well-defined 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD (Dauer and Przedborski, 2003; Vila and Przedborski, 2003). Recently, the loss of parkin function has also been implicated in neuronal death in this model (Chung et al., 2004; Yao et al., 2004). We found that the targeted expression of BAG5 in the SNpc of mice by Ad.BAG5 (Figure 7E) significantly enhanced the loss of dopaminergic neurons in the SNpc ipsilateral to the intrastriatal viral injection at 2 weeks following MPTP administration when compared to those animals injected with the control virus Ad.GFP (Figures 7F and 7G). Next, we examined the effect of mutant BAG5(DARA) (Figure 7E), which we have shown to bind to and mitigate the inhibitory effects of BAG5 on Hsp70 and parkin function (Figures 3E–3G, 5D, and 6D–6E). We found that targeted expression of BAG5(DARA) resulted in a significant increase in dopaminergic neuronal survival (Figures 7F and 7G). Therefore, we conclude that targeted expression of BAG5 in the SNpc enhances dopaminergic neurodegeneration, whereas targeted ex-

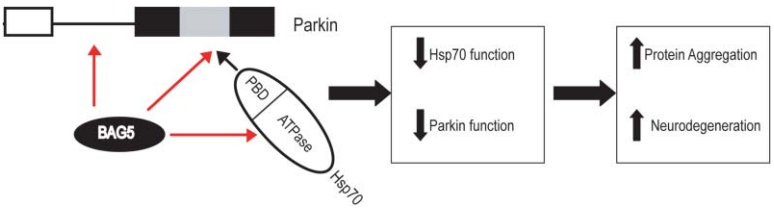

Figure 8. Proposed Model for the Role of BAG5 in Enhancing Neurodegeneration

pression of the inhibitory mutant BAG5(DARA) in the SNpc suppresses dopaminergic neurodegeneration in an in vivo model of PD.

Discussion

We have demonstrated that BAG5, a BAG family member whose expression is induced following dopaminergic neuron injury, is a negative regulator of both Hsp70 and parkin function that sensitizes dopaminergic neurons to injury-induced death. The exact mechanism by which chaperones such as Hsp70 confer neuroprotection in models of neurodegeneration is not yet clear. Overexpression of Hsp70 can prevent the formation of protein aggregates and enhance cell survival in models of polyglutamine (polyQ) expansion-mediated neurodegeneration in vivo (Adachi et al., 2003; Chan et al., 2000). In models of PD (Auluck et al., 2002; Klucken et al., 2004) and certain cases of polyQ expansion-mediated neurodegeneration (Cummings et al., 2001; Warrick et al., 1999), neuroprotection by Hsp70 can also occur without affecting protein aggregation. In these latter cases, it is conceivable that inhibition of cell death occurs by Hsp70 directly inhibiting cell death mediators (Takayama et al., 2003). Here, we show that BAG5 but not BAG5(DARA) interacts directly with Hsp70, preventing the refolding of misfolded proteins and protein aggregation in cells. Consistent with this finding, the BAG5-Hsp70 interaction is likely to have important functional consequences in vivo, since we observed enhanced cell death in dopaminergic neurons overexpressing BAG5.

A significant proportion of early-onset cases of PD are associated with mutations that result in the loss of parkin E3 function (Imai et al., 2000, 2001; Shimura et al., 2000; Zhang et al., 2000). In these cases, it is likely that the loss of parkin function leads to the accumulation of parkin substrates and subsequent UPS dysfunction leading to neurodegeneration, often in the absence of protein aggregates (Giasson and Lee, 2003). Furthermore, S-nitrosylation-mediated inhibition of parkin activity has recently been shown to be a potential link between the genetic and sporadic forms of PD (Chung et al., 2004; Yao et al., 2004). Indeed, depression of parkin expression in the context of substrate accumulation enhances dopaminergic cell death (Petrucelli et al., 2002; Yang et al., 2003), whereas overexpression of parkin is often neuroprotective (Imai et al., 2000, 2001; Petrucelli et al., 2002; Yang et al., 2003). Here, we show that BAG5 interacts directly with parkin and that it may act as a negative modulator of parkin, attenuating the parkin-mediated preservation of UPS function and cell survival. The exact mechanism by which BAG5 inhibits parkin E3 activity remains to be elucidated. One possibility is that BAG5 inhibits parkin by binding and blocking the RING finger domains at the C terminus of parkin, interfering with its interaction with E2s. A second possibility is that BAG5 may induce conformational changes in parkin that result in the inhibition of E3 activity.

The BAG domain consists of three amphiphatic α helices (Briknarova et al., 2001; Sondermann et al., 2001). Recent models of interactions with the BAG domain, based on both structure and function analysis (Brehmer et al., 2001; Briknarova et al., 2001, 2002; Brive et al., 2001; Sondermann et al., 2001, 2002), suggest that Hsp70 binding occurs at the second and third α helices, whereas other BAG domain binding partners bind to the first and second α helices (Takayama and Reed, 2001). Thus, binding partners may "compete" for BAG domain binding based on their relative availability (Nollen and Morimoto, 2002; Song et al., 2001). Once binding occurs, the BAG protein may modify the function of its target protein, as we have demonstrated with the BAG5 inhibition of both parkin and Hsp70.

The role of protein aggregation in the initiation and progression of neurodegenerative disease remains controversial. Indeed, the sequestration of misfolded proteins into aggregates may initially be part of a protective cellular response to transient imbalances in protein folding and degradation pathways (Sherman and Goldberg, 2001). However, the expression of defective proteins in familial forms of neurodegenerative disease or the targeted depression of parkin expression imposes protracted exposure to abnormal conditions which can result in direct inhibition of the UPS (Bence et al., 2001; Petrucelli et al., 2002). Similarly, we show that the accumulation of the parkin substrate synphilin can result in proteasome dysfunction and that BAG5 can enhance proteasome dysfunction. This feed-forward cycle of UPS inhibition alone may be sufficient to perturb protein homeostasis and enhance the sensitivity of cells to subsequent stress. As aggregates persist and additional proteins such as parkin and Hsp70 are increasingly mislocalized and thus less able to effectively fulfill their roles in neuroprotection, the likelihood of cell death increases.

In addition to these formidable liabilities, our studies implicate BAG5 as a participant in protein aggregation. BAG5 enhances the formation of parkin-containing protein aggregates, probably through BAG5-mediated inhibition of Hsp70 chaperone activity, and overrides the ability of parkin to preserve proteasome function, whereas the inhibitory mutant BAG5(DARA) can prevent this effect. Given that BAG5(DARA) retains the ability to associate with wild-type BAG5, our data suggest that the mechanism of BAG5(DARA) inhibition is likely through the neutralization of available BAG5. However, we cannot rule out the possibility of other contributing mechanisms, since BAG5(DARA) may retain regulatory activity

on those BAG5 binding proteins that interact with the first α helix of each BAG domain. Our observation that the depression of endogenous BAG5 expression by shRNA leads to decreased protein aggregation and synphilin/α-synuclein-mediated toxicity in cells further supports the possibility that BAG5 plays an important role in the regulation of aggregate biogenesis and cell death. Although the exact role of protein aggregates in neurodegeneration associated with PD has not yet been established, BAG5 may serve as an important link between the chaperone system, the UPS, and protein aggregates, by forming multiprotein regulatory complexes with Hsp70 and parkin similar to parkin-containing SCF-like ubiquitin ligase complexes (Staropoli et al., 2003). BAG1 and BAG3, two other members of the BAG family, have also been recently characterized as potential links between chaperones and the UPS (Alberti et al., 2002; Demand et al., 2001; Doong et al., 2003; Luders et al., 2000).

Finally, we show that targeted expression of BAG5 within the SNpc enhances dopaminergic neurodegeneration in vivo in an axotomy model of dopaminergic neuron injury and in the MPTP model of PD. We also show that BAG5(DARA), an inhibitory mutant of BAG5, confers significant neuroprotection in the MPTP model of PD, thus suggesting that BAG5 may play an important role in the loss of dopaminergic neurons. It still remains to be determined whether the enhanced neurodegeneration observed in this study is a unique function of BAG5 or if other BAG domain-containing proteins can also act as prodeath molecules in the SNpc. Recent evidence implicates other BAG family proteins as molecular sensors that can enhance cell survival by interacting with available substrates in diverse signaling pathways (Song et al., 2001; Takayama and Reed, 2001). Indeed, transgenic overexpression of BAG1 in mice has been shown to confer neuroprotection in an animal model of stroke (Kermer et al., 2003).

How BAG5 may differ from other BAG family members and how BAG5 may contribute to the selective sensitivity of dopaminergic neurons will require further investigation. If the chaperone response is impaired in stressed dopaminergic neurons as it may be in other neurodegenerative processes (Batulan et al., 2003), it is possible that the relative amount of available BAG5 may be increased. Indeed, the levels of Hsp70, BAG protein, and BAG protein substrates have already been shown to have significant biological consequences (Song et al., 2001). Loss of parkin function, through mutation as in AR-JPD (Kitada et al., 1998) or nitrosylation (Chung et al., 2004; Yao et al., 2004), results in dopaminergic neurodegeneration. Since BAG5 inhibits parkin activity, it is conceivable that increases in the relative amount of available BAG5, by either increased expression or impaired Hsp70 availability, may contribute to selective dopaminergic neurodegeneration. Based on our findings, we propose a mechanism for neurodegeneration in which BAG5 can interact with both parkin and Hsp70, resulting in decreased parkin and Hsp70 function, two outcomes that are deleterious to cell survival (Figure 8). Given the role of BAG5 in modulating ubiquitinylation, protein aggregation, and cell death, it may serve as a useful therapeutic target for neurodegenerative diseases such as PD.

Experimental Procedures

All reagents were used according to manufacturer's protocol unless otherwise stated.

Cloning

Cloning of BAG5 and other constructs is outlined in the Supplemental Data at http://www.neuron.org/cgi/content/full/44/6/931/DC1/.

Northern Blots

mRNA Northern blots (Origene) were probed with rat or human Bag5 cDNA or β-actin (Ambion) labeled with [^{32}P]ATP (NEN) using the StripEZ probe NorthernMax-Gly kits (Ambion).

ISH, IHC, and Immunocytochemistry

IHC was performed as we have previously described (Crocker et al., 2001). Rat BAG5 probes for ISH were labeled using [^{33}P]UTP (NEN) or digoxygenin (DIG) (Roche) via in vitro transcription with SP6 or T7 RNA polymerase (Promega). Immunocytochemistry (ICC) was performed using conditions described by Junn et al. (2002) and Chung et al. (2001), and cells were analyzed using confocal microscopy (LSM-510 Meta; Zeiss).

Antibodies

Tyrosine hydroxylase (Immunostar), α-synuclein (BD Bioscience), Hsp70 (monoclonal; Stressgen), FLAG (M2, Cy3-M2, and polyclonal; Sigma), His-tag (Amersham), HA-tag (Roche), ubiquitin (Chemicon), parkin (Cell Signal), GAPDH (Chemicon), β-galactosidase (Promega), and GFP (polyclonal; Molecular Probes). BAG5 polyclonal antibodies were generated by Exalpha Biologicals against KLH-conjugated BAG5(118-134) peptide (Dalton). Affinity-purified antibody was generated using a sulfhydryl coupling column (Sulfolink Kit; Pierce).

Cell Culture

HEK293T, NIH 3T3, and SH-SY5Y cells were maintained in DMEM growth medium supplemented with 10% fetal bovine serum, 0.5 mg/ml amphotericin B, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2.

Fusion Proteins

All GST and His fusion proteins were expressed in *E. coli* and affinity purified using Glutathione Sepharose 4B beads and HiTrap Columns (Amersham), respectively. His-Ubc6ΔC (C-terminal truncated Ubc6) and His-Ubc7 (Imai et al., 2001) (kind gift of R. Takahashi) were affinity purified using Ni-NTA Agarose (Qiagen).

PDAs and IPs

Samples were harvested in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (Roche). For complete solubilization of the pellet in synphilin ubiquitinylation experiments, boiling lysis buffer with 1% SDS was used. IPs were performed overnight followed by incubation with Protein G sepharose beads (Amersham). PDAs were performed with equimolar amounts of GST and His fusion proteins for 1 hr (4°C). Beads were washed once with lysis buffer and three times with PBS (pH 7.4).

Luciferase Refolding Assay

Refolding assays were performed as described by Nollen et al. (2001) using indicated vectors and normalized for transfection efficiency by β-galactosidase activity (pSV-β-Galactosidase Control Vector; Promega).

Parkin Ubiquitinylation Assays

Ubiquitinylation was carried out by incubating 2 μg GST or GST-Parkin, 3 μg His-BAG5, 3 μg His-Hsp70, with mammalian E1 ubiquitin-activating enzyme (Boston Biochem) (180 nM), the E2 ubiquitin-conjugating enzymes Ubc6ΔC (2 μg) and Ubc7 (2 μg) (kind gift of R. Takahashi), and 20 μM Ub (Sigma), in 50 mM Tris (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 μM Ubiquitin Aldehyde (Boston Biochem), and 4 mM ATP. The reaction was incubated at 37°C for 45 min and resolved by SDS-PAGE. Synphilin ubiquitinylation assays were performed in HEK293T cells as described by others

(Chung et al., 2001, 2004). Cells were cotransfected with indicated vectors and HA-ubiquitin (kind gift of J. Wrana) and treated with 20 μM of the proteasome inhibitor MG132 for 6 hr prior to harvesting.

Parkin Sequestration

Parkin sequestration assays were performed as described by Junn et al. (2002) with GFP-parkin and modifications described in the text.

shRNA-Mediated Knockdown of BAG5

Vectors for U6-driven expression of shRNAs were generated from a U6 promoter-containing vector template (kind gift of G. Hannon) (for primers, see the Supplemental Data at http://www.neuron.org/cgi/content/full/44/6/931/DC1/).

GFPu Proteasome Reporter Assays

The GFPu reporter construct was cotransfected with the indicated vectors in HEK293T cells and lysed at 48 hr using gel loading buffer. GFPu accumulation was measured by fluorescence microscopy and WB quantitation relative to transfection controls (DsRed and β-galactosidase).

Cell Death Analysis

Similar to others (Chung et al., 2004), we transfected SH-SY5Y cells with the indicated plasmids for 24 hr followed by treatment with lactacystin for 18 hr, and cell death was quantified with Hoechst 33342 (Molecular Probes) and propidium iodide (Molecular Probes).

Intrastriatal Adenovirus Injection, MFB Axotomy, and the MPTP Model of PD

Intrastriatal adenovirus injections were performed in rats and mice as we have previously described (Crocker et al., 2001; Smith et al., 2003) in compliance with the Canadian Council on Animal Care guidelines. Adenovirus was generated by subcloning FLAG-BAG5 and FLAG-BAG5(DARA) into AdTrack-CMV using the AdEasy system (Quantum). Ad.GFP was generated using empty AdTrack-CMV shuttle vector. MFB axotomy in rats, MPTP injections in mice, and analysis were performed as we have previously described (Crocker et al., 2001; Smith et al., 2003).

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Accession Numbers

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